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A Coupled Microsomal-Activating/Embryo Culture System: Toxicity of Reduced beta-Nicotinamide Adenine Dinucleotide Phosphate (NADPH)

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16. ABSTRACT

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A COUPLED MICROSOMAL-ACTIVATING/EMBRYO CULTURE SYSTEM: TOXICITY OF REDUCED β-NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE (NADPH)*

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Abstract—An NADPH-dependent microsomal-activating system has been coupled to a rat embryo culture *in vitro*. No embryonic morphological abnormalities or decreases in final yolk sac or embryo DNA and protein contents occurred when 0.2 mM NADPH was used in this coupled system. In contrast, 1.0 mM NADPH alone, or 0.2 mM NADPH in the presence of microsomes and a glucose-6-phosphate dehydrogenase-based NADPH-generating system, greatly reduced embryo and yolk sac growth *in vitro*. The toxicity of NADPH was not due to lipid peroxidation. Only minor decreases in final yolk sac protein levels occurred when embryos were grown in media containing male rat microsomes and 1.0 mM NADPH. The protective effect of rat hepatic microsomes on NADPH toxicity does not seem to have been due to the oxidation of NADPH to the less toxic NADP. Although cyclophosphamide alone was not toxic to rat embryos cultured *in vitro*, in the coupled microsomal-activating/embryo culture system, cyclophosphamide reduced yolk sac and embryo growth and caused abnormal embryonic differentiation. The uses of the coupled microsomal-activating/embryo culture system to study mechanisms in anomalous development, as well as its possible use in embryo toxicity and teratogenicity testing, are discussed.

Rat conceptuses (embryo plus yolk sac and some attached placenta) of pregnancy day 11 can be cultured *in vitro* for 48 hr with heat-inactivated male rat serum [1, 2]. During this period of organogenesis, the embryo DNA and protein contents increase by 14- and 12-fold respectively. Increases of 8- and 11-fold are observed in the yolk sac DNA and protein levels during this time period. Furthermore, extensive differentiation of the vital organs of the embryo occurs during the day 11–13 period. Embryonic growth and differentiation resemble that *in utero* during the same period of development [1]. The *in vitro* embryo culture system could be a useful model for studying embryo toxicity or teratogenesis [3]. Although the embryo culture system is highly sensitive to the effects of direct alkylating agents such as 2,4,6-triethylenimino-1,3,5-triazine [4, 5], the embryo has little or no ability to biotransform inactive compounds to more reactive metabolites via mechanisms such as the microsomal mixed-function oxidase enzyme system. Therefore, it is desirable to increase the metabolizing ability of *in vitro* embryo culture by adding adult rat liver microsomes. The addition of adult metabolic activating systems to conceptus culture should increase the versatility and sensitivity of this *in vitro* embryo toxicity/teratogenicity technique. Such an addition will also be useful in assessing the importance of maternal metabolism to embryo toxicity and teratogenesis.

In this study, optimal conditions for adding a rat hepatic microsomal activating system to an *in vitro* conceptus culture system have been determined. The effects of NADPH, uninduced male rat hepatic microsomes, and a glucose-6-phosphate dehydrogenase-based NADPH-generating system on embryonic and yolk sac growth were determined. Experiments were performed to determine if the embryo and yolk sac toxicity of NADPH was due to NADPH-induced lipid peroxidation and if microsomes protected against NADPH toxicity by oxidizing it to non-toxic NADP. Finally, the effect of cyclophosphamide (a teratogen which may require metabolic activation to express its biological activity) was determined in the coupled microsomal-activating/embryo culture system. This result is compared to the effects on conceptus growth of aminopyrine, a non-teratogen, and of the direct-acting alkylating agent nitrogen mustard, which is structurally related to cyclophosphamide.

METHODS

Isolation of liver microsomes. Microsomes were isolated from adult (Charles River CD-strain) male rat (200–280 g) liver homogenized in 1.15% KCl containing 0.02 M Tris buffer, pH 7.5. The microsomal

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pellet was obtained from the 9000 *g* supernatant fraction as described previously [6], suspended, recentrifuged, and finally homogenized in cold rat serum.

Conceptus culture and treatment groups. The day that sperm were found in the vaginal smear was designated day 1. Rat conceptuses of pregnancy day 11 (embryonic age 10.5 days) were cultured for 48 hr in rotating 30 ml serum bottles, according to the methods described earlier [7, 8]. They were grown in fresh 100% serum obtained from 65- to 75-day-old male rats. The incubation serum contained streptomycin (50 μg/ml) and penicillin (50 I.U./ml) (GIBCO). Each conceptus was cultured in 5 ml of fresh serum medium gassed with 20% O_2, 5% CO_2, and 75% N_2 for 20 hr and with 40% O_2, 5% CO_2, and 55% N_2 for the next 28 hr [8]. Conceptuses with embryos rotated into concave fetal position (Witschi state 16), collected from several rats, were randomly distributed to the various treatment groups. At the end of the 48-hr culture period, conceptuses were removed from the medium and washed in 5 ml of Ringer's solution. The diameter of the yolk sac, beating of the heart, and number of somites were noted, using a binocular stereomicroscope. The appearances of anatomical anomalies in the embryos were assessed visually. On the basis of microscopic observation, embryos were classified as either normal or abnormal when compared to untreated embryos cultured *in vitro*. The term "abnormal embryo" includes both severe growth retardation, which prevents normal *in vitro* differentiation, as well as specific dysmorphogenesis. The embryo and the yolk sac, dissected from each conceptus, were frozen in liquid nitrogen and stored at −15° until utilized within 2–3 weeks for DNA and protein measurements. These tissues were homogenized in 1 ml of ice-cold 0.05 M phosphate buffer, pH 7.4, with a sonifier (Branson Sonic Power Co.). DNA was estimated by the ethidium bromide fluorescence method after digestion of aliquots of homogenate with RNase and protease [9]. Protein was measured using the method of Lowry *et al.* [10]. The DNA and protein levels are expressed on a per embryo and yolk sac basis.

Chemicals, biochemicals, assays, and statistics. NADPH, NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from the Sigma Chemical Co. (St. Louis, MO). Nitrogen mustard hydrochloride, cyclophosphamide, and aminopyrine were obtained from ICN Pharmaceuticals (Cleveland, OH), Mead & Johnson (Evansville, IN), and the Aldrich Chemical Co. (Milwaukee, WI) respectively. The oxidation of NADPH was determined spectrophotometrically by the decrease in absorption at 340 nm. Formation of malondialdehyde at 37° from rat hepatic microsomes suspended in 0.05 M Tris–HCl buffer or rat serum was terminated by the addition of 0.2% butylated hydroxytoluene. The malondialdehyde formed was determined by heating with 2-thiobarbituric acid and measuring the absorption at 535 nm, using an extinction coefficient of $1.56 \times 10^5\ M^{-1}$ [11]. *N*-Demethyl-

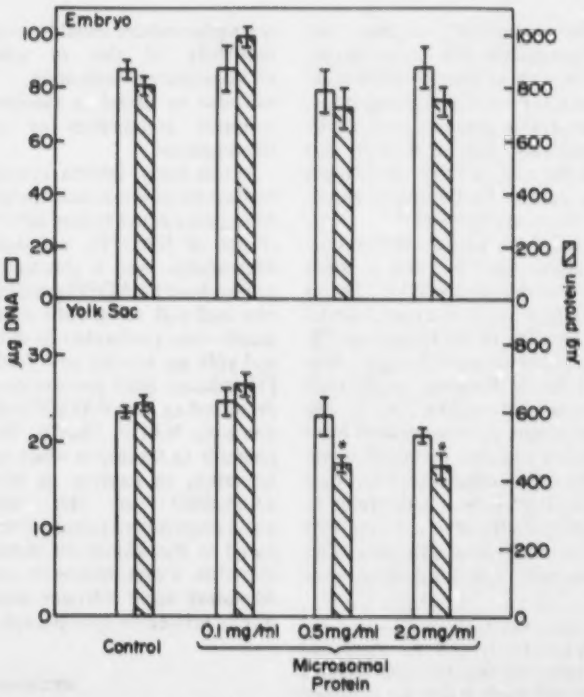

Fig. 1. Effect of microsomes on rat conceptus growth *in vitro*. Day 11 rat conceptuses were cultured for 46–48 hr in heat-inactivated rat serum containing 0.1, 0.5, or 2.0 mg of microsomal protein/ml. Microsomes were obtained from uninduced adult male rat liver. Each final DNA and protein level is the mean ± S.E.M. for four or more conceptuses. Values significantly different ($P < 0.001$) from control DNA and protein levels are indicated with an asterisk.

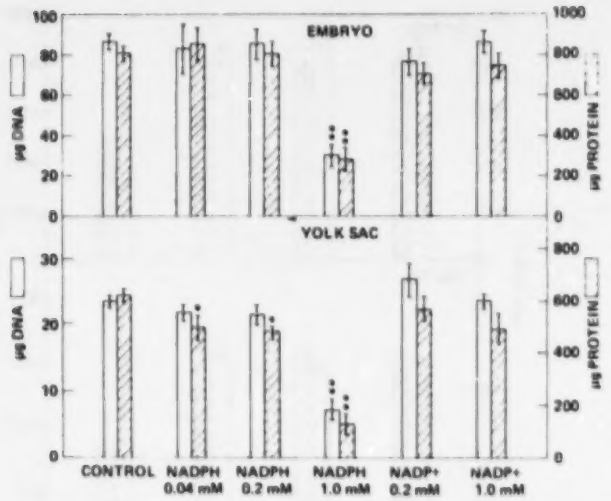

Fig. 2. Effects of NADPH and NADP on rat conceptus growth *in vitro*. Day 11 rat conceptuses were cultured for 46–48 hr in rat serum containing the indicated concentrations of NADPH or NADP. Each final DNA and protein level is the mean ± S.E.M. for five or more conceptuses. Values statistically different from controls are indicated by one ($P < 0.01$) or two ($P < 0.001$) asterisks.

ation of aminopyrine was determined by the method of Nash [12]. Assay conditions were as described by Lucier *et al.* [13].

Quantitative data are presented as the mean ± S.E.M. for each treatment group; significance was evaluated using analysis of variance and Student's *t*-test [14]. Enumerative data are expressed as the incidence of occurrence, and the statistical significance was calculated by using chi-square contingency tables (exact method) [15].

RESULTS

To couple a microsomal-activating system to embryo culture *in vitro*, the effects of the components of the microsomal-activating system on conceptus growth and differentiation were first determined. The effects of increasing amounts of microsomal protein (0.1 to 2.0 mg/ml) on final embryo and yolk sac DNA and protein contents are shown in Fig. 1. The addition of male rat hepatic microsomal protein to the embryo culture media had no effect on final embryo DNA and protein contents. Although microsomal protein did not decrease yolk sac DNA levels, the final yolk sac protein contents of conceptuses grown with 0.5 and 2.0 mg/ml of microsomal protein were both significantly ($P < 0.001$) depressed to 71 per cent of control values.

Because of its embryo-lethal properties, cumene hydroperoxide could not be used to drive microsomal cytochrome P-450-dependent reactions in this *in vitro* embryo culture. Embryo growth, as reflected in its DNA and protein contents, was not affected by 0.2 or 1.0 mM NADP or by 0.04 or 0.2 mM NADPH (Fig. 2). However, 1.0 mM NADPH greatly inhibited ($P < 0.001$) increases in embryonic DNA and protein contents during the 48-hr incubation period. Because the serum medium contained about 75 mg protein/ml, substantial protein binding of NADPH may have occurred, thus requiring high NADPH concentrations for high rates of NADPH-dependent cytochrome P-450-based catalysis to occur. Embryos exposed to these high concentrations of NADPH grew to have only 35 per cent of the expected DNA and protein values. The yolk sac DNA and protein contents of conceptuses grown with 1.0 mM NADPH in male rat serum were only 30 and 21 per cent of control values. NADPH (0.04 to 0.2 mM) or NADP (0.2 to 1.0 mM) had no significant effects on yolk sac DNA levels. The final yolk sac protein concentration was reduced by about 20 per cent in the conceptuses grown in either 1.0 mM NADP, 0.04 mM NADPH, or 0.2 mM NADPH.

Because of the toxicity of 1.0 mM NADPH to embryos, experiments were performed to determine if the glucose-6-phosphate dehydrogenase-based NADPH-generating system could be used with 0.2 mM NADPH to provide a long-lasting source of NADPH to drive the cytochrome P-450-dependent microsomal mixed-function oxidase system in the serum medium. Results of experiments are shown in Fig. 3. The generating system of glucose-6-phosphate (10 mM) and glucose-6-phosphate dehydrogenase (1 unit/ml), either alone or in combination with 0.2 mM NADPH, did not affect embryo growth or differentiation *in vitro*. The addition of male rat hepatic microsomes (0.5 mg/ml) to 0.2 mM NADPH and the NADPH-generating system, however, did not lead to a successful coupled microsomal-activating/embryo culture system. The DNA contents of embryos and yolk sacs increased to only 60 per cent of control values in this medium. The protein contents of embryos and yolk sacs grown in serum containing an NADPH-generating system, 0.2 mM NADPH, and microsomes (0.5 mg protein/ml) were only 50 per cent of control values. Similar unsuc-

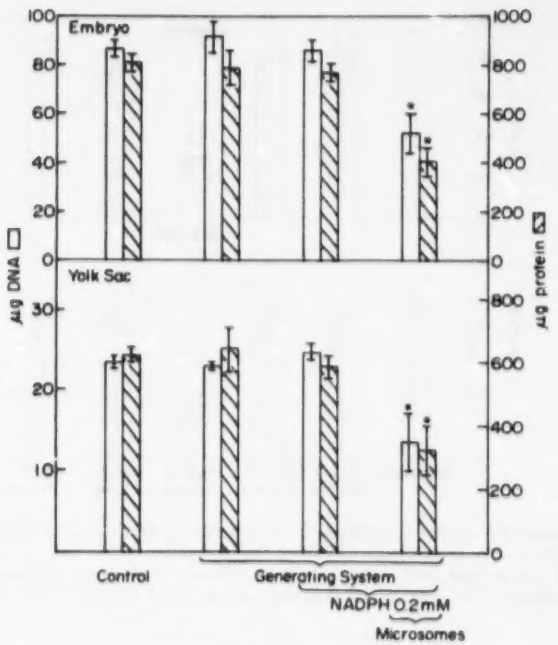

Fig. 3. Effect of a glucose-6-phosphate-based NADPH-generating system on rat conceptus growth *in vitro*. Day 11 rat conceptuses were cultured in rat serum containing an NADPH-generating system (10 mM glucose-6-phosphate and 1 unit/ml glucose-6 phosphate dehydrogenase), 0.2 mM NADPH, and 0.4 to 0.5 mg rat hepatic microsomal protein per ml as indicated. Each value is the mean ± S.E.M. for four or more conceptuses. An asterisk indicates a statistically significant difference from controls at the $P < 0.001$ level.

Table 1. Structural and functional assessment of embryos cultured for 46–48 hr in serum media containing metabolic activating components*

Treatment	Yolk sac diameter (mm)	Somite number in embryo	Embryos with heartbeat	Abnormal embryos
Control	7.46 ± 0.15 (18)	38.4 ± 0.36 (18)	18/18	0/18
1.0 mM NADPH	2.85 ± 0.68† (14)	27.4 ± 2.01† (7) NC‡ (7)	3/13†	11/14†
Microsomes (0.5 mg protein/ml)	7.56 ± 0.09 (5)	39.0 ± 0.32 (5)	5/5	0/5
0.2 mM NADPH microsomes (0.5 mg protein/ml)	7.88 ± 0.18 (6)	39.3 ± 0.33 (6)	6/6	0/6
Generating system	7.47 ± 0.28 (5)	38.6 ± 0.51 (5)	5/5	0/5
0.2 mM NADPH generating system	7.52 ± 0.20 (8)	36.6 ± 0.69§ (7)	8/8	3/7§
0.2 mM NADPH microsomes (0.5 mg protein/ml) generating system	4.64 ± 0.33† (6)	25.0 ± 3.0† (2) NC‡ (3)	1/4§	6/6†

* Day 11 rat conceptuses were cultured in heat-inactivated rat serum with the indicated additions as described in Methods. Microsomes were obtained from uninduced male rat liver. The NADPH-generating system consisted of 10 mM glucose-6-phosphate and 1 unit/ml of glucose-6-phosphate dehydrogenase.
† $P < 0.001$, compared to control.
‡ Not countable.
§ $P < 0.05$, compared to control.

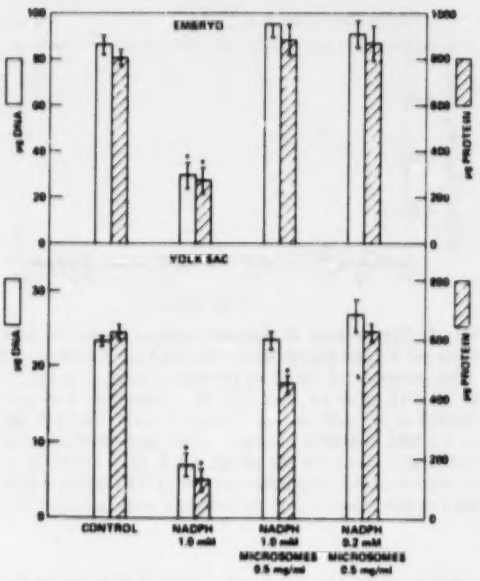

Fig. 4. Development of a coupled microsomal-activating/embryo culture system. Day 11 rat conceptuses were cultured for 46–48 hr in heat-inactivated rat serum containing the indicated additions. Each final DNA and protein value is the mean ± S.E.M. for five or more conceptuses. An asterisk indicates a statistically significant difference from controls at the $P < 0.001$ level.

cessful results were obtained with an isocitrate dehydrogenase-based NADPH-generating system.

The effects of 1.0 mM NADPH and also of 0.2 mM NADPH, microsomes, and an NADPH-generating system on embryonic differentiation were determined (Table 1). None of these alone, or NADPH plus microsomes, diminished yolk sac diameters, number of embryo somites, heart function, or morphological integrity of the embryos. Conceptuses cultured in serum medium containing 0.2 mM NADPH and a generating system had normal yolk sac diameters and heart function, but they had slightly smaller numbers of embryonic somites and a noticeably elevated incidence of abnormal embryos. The presence of either 1.0 mM NADPH or 0.2 mM NADPH, a generating system, and microsomes significantly reduced the yolk sac diameters, the number of embryo somites, and heart function. Most of the embryos in these two groups were structurally abnormal. Snout, optic, otic, and branchial arch abnormalities were the most frequently observed malformations in conceptuses grown in serum containing 0.2 mM NADPH, microsomes, and an NADPH-generating system.

Because an NADPH-generating system could not be included in our embryo culture system, experiments were performed to determine if NADPH and microsomes would be a satisfactory microsomal activating system. Results of these studies are presented in Fig. 4. Although 1.0 mM NADPH was highly toxic to embryo growth *in vitro*, this effect was completely abolished by including 0.4 to 0.5 mg/ml of rat hepatic microsomal protein in the serum media. This combination allowed normal yolk sac DNA synthesis, although the final yolk sac protein levels were significantly lower ($P < 0.001$, 73 per cent of control levels). Even this small decrement in yolk sac growth could be abolished if rat conceptuses was cultured with a lower concentration of NADPH (0.2 mM) with microsomes. Embryos grown in medium containing microsomes and 0.2 to 1.0 mM NADPH differentiated normally. Thus, the combination of either 0.2 or 1.0 mM NADPH and microsomes (0.5 mg protein/ml) fulfilled the requirement for a microsomal-activating system which did

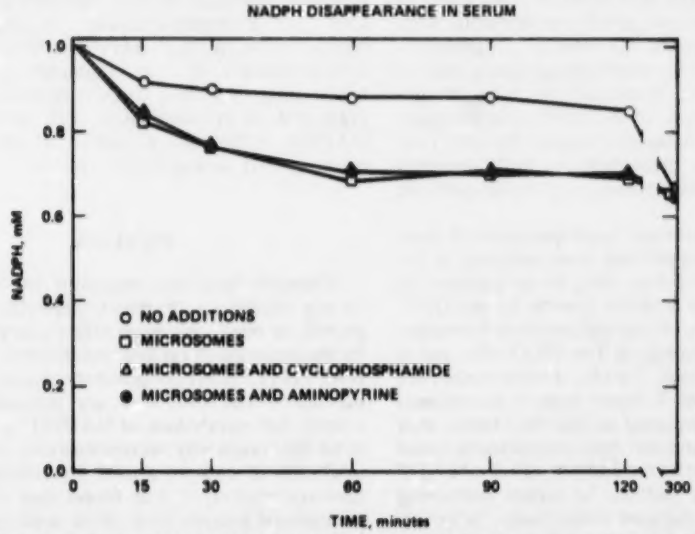

Fig. 5. Disappearance of NADPH in heat-inactivated serum incubation media. NADPH concentrations were determined by measuring the absorption at 340 nm at various times after a 37° incubation under a 5% CO_2, 20% O_2, and 75% N_2 gas phase was begun. Values are means of four determinations per point.

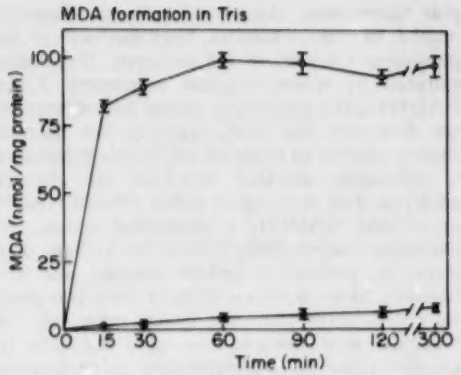

Fig. 6. Time course of malondialdehyde formation from adult rat hepatic microsomes (0.4 to 0.5 mg protein/ml) in 0.05 M Tris–HCl buffer, pH 7.5. Incubations at 37° were done with 0.2 mM NADPH (●) or with 0.2 mM NADPH, 1.0 mM ADP, and 10 μM ferrous sulfate (○). Data are the means ± S.E.M. for four determinations done in duplicate (per point).

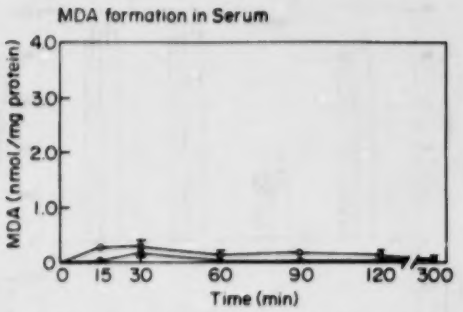

Fig. 7. Time course of malondialdehyde formation from adult rat hepatic microsomes (0.4 to 0.5 mg protein/ml) in serum medium (about 75 mg protein/ml) under a gas phase of 5% CO_2, 20% O_2, and 75% N_2. Incubations were performed at 37° with the addition of 0.2 mM NADPH (●) or 0.2 mM NADPH, 1.0 mM ADP, and 10 μM ferrous sulfate (○). Data are the means ± S.E.M. for four determinations done in duplicate (per point). The ordinate scale used in this figure is 1/25 the ordinate scale used in Fig. 6.

not *per se* adversely affect embryo growth or differentiation.

To determine if this microsomal-activating system could bioactivate chemicals in the serum medium, experiments were performed to compare the rates of *N*-demethylation of aminopyrine in serum containing 1.0 mM NADPH and in Tris–HCl buffer. The microsomal mixed-function oxidase enzyme activity in serum medium with a 20% O_2, 5% CO_2, and 75% N_2 gas phase in rotating roller bottles was 83 per cent of the enzyme activity observed with microsomes suspended in 0.05 M Tris–HCl buffer, pH 7.5, in air [7.31 nmoles formaldehyde·(mg microsomal protein)$^{-1}$·min^{-1}].

Because the addition of hepatic microsomes to serum culture medium abolished the toxic effect of NADPH on embryonic growth, experiments were performed to determine the rates of NADPH disappearance in serum containing microsomes and two different mixed-function oxidase substrates. No differences in the rates of NADPH disappearance occurred in serum containing microsomes (or Tris–HCl buffer, data not presented) due to the presence of the substrates aminopyrine or cyclophosphamide (Fig. 5).

Because serum contains large quantities of iron, lipid peroxidation might have been occurring in the presence of NADPH and, thus, be an explanation of the inhibition of embryo growth by NADPH. Therefore, the rates of malondialdehyde formation of rat hepatic microsomes in Tris–HCl buffer and in serum were determined. Results of these studies are shown in Figs. 6 and 7. Some hepatic microsomal lipid peroxidation occurred in Tris–HCl buffer with 0.2 mM NADPH, and this lipid peroxidation could be greatly stimulated by the addition of 1.0 mM ADP and 10 μM ferrous sulfate. In serum containing microsomes, or in cultured conceptuses, however, the malondialdehyde formation rate was not stimulated by the addition of ADP and ferrous sulfate. Neither serum NADPH disappearance rates nor malondialdehyde production rates were greatly influenced by the presence of the conceptus in the serum medium.

Nitrogen mustard (1 μg/ml) in the incubation medium greatly inhibited embryo and yolk sac growth (Fig. 8). However, cyclophosphamide, an organophosphorus compound that may require *in vivo* activation to be teratogenic, did not inhibit embryo and yolk sac growth *in vitro*. When cyclophosphamide, rat hepatic microsomes, and NADPH were all present in the serum incubation media, however, the embryonic DNA and protein values increased to only 9 and 23 per cent of the controls respectively. Organogenesis and differentiation of the embryo were adversely affected in this treatment group [16]. *In vitro* yolk sac growth was also significantly inhibited, further demonstrating the toxicity of cyclophosphamide in the coupled microsomal-activating/embryo culture system. In similar experiments, an equimolar quantity of a non-teratogen, aminopyrine, either alone (data not presented) or in combination with microsomes and NADPH in the culture media, did not affect conceptus growth and development.

DISCUSSION

Although high concentrations of NADPH are clearly inhibitory to post-implantation conceptus growth *in vitro*, this toxic effect can be prevented by the presence of rat liver microsomes. The rate of NADPH (1.0 mM) disappearance in serum containing microsomes is too slow, and plateaus at too high a level, for metabolism of NADPH by microsomes to be the reason why microsomes can antagonize the inhibition of embryo growth by NADPH. In preliminary studies, it was found that 0.5 mg/ml of microsomal protein from either adult or fetal liver, kidney, or spleen antagonized the toxicity of 1.0 mM NADPH, but the same concentration of rat brain microsomes did not have this effect (data not presented). Our evidence indicates that appreciable

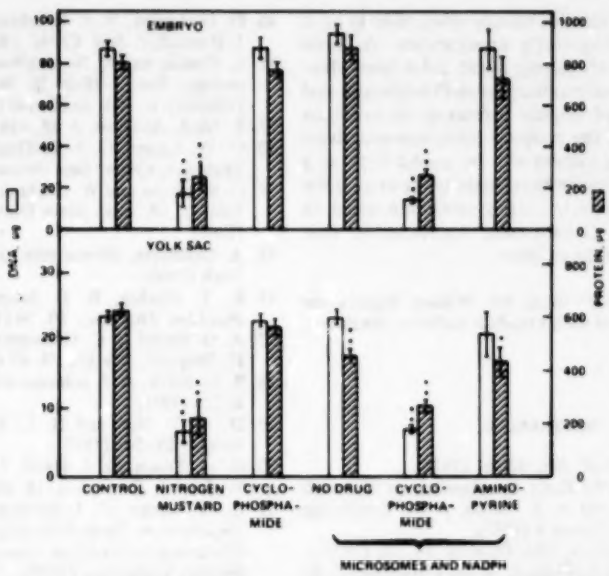

Fig. 8. Effects of nitrogen mustard (1 μg/ml) on conceptus growth and of cyclophosphamide (100 μg/ml) and aminopyrine (80 μg/ml) on conceptus growth in medium containing microsomes (0.4 to 0.5 mg/ml) and 1.0 mM NADPH. Day 11 rat conceptuses were cultured for 46–48 hr. Each final DNA and protein level is the mean ± S.E.M. for four or more conceptuses. Values statistically different from controls are indicated with one ($P < 0.01$) or two ($P < 0.001$) asterisks.

lipid peroxidation did not occur in the serum containing hepatic microsomes even with added ferrous iron.

The toxicity of the glucose-6-phosphate-generating system was quite different from that of NADPH alone. NADPH was toxic only without microsomes; NADPH plus the generating system was most toxic with microsomes present in the serum incubation media. It is unclear at this time whether the glucose-6-phosphate-based NADPH-generating system was toxic because it maintained the 0.2 mM concentration of NADPH for longer times or if the generating system allowed the microsomes to be functionally operative for a longer period of time. Either could have depleted the serum of the chemicals needed for conceptus growth or have generated toxic metabolites of serum components.

These studies indicate that including an adult hepatic metabolic activating system in the conceptus culture medium can improve versatility and sensitivity. This system uses control male rat hepatic microsomes (0.35 to 0.5 mg protein/ml) and NADPH in concentrations from 0.2 to 1.0 mM. There are several advantages of this microsomal-activating/embryo culture system compared to that recently described by Fantel *et al.* [17], who used post-mitochondrial supernatant fractions from Aroclor 1254-pretreated rats with conceptuses cultured in heat-inactivated human serum. Immunological problems may exist when rodent conceptuses are cultured in human serum. For example, rat yolk-sac antiserum is known to be teratogenic *in vivo* [18], as well as *in vitro* [19]. Aroclor 1254, a mixture of many chlorinated biphenyls, also has been found to be teratogenic in animals. 3,4,3′,4′-Tetrachlorobiphenyl, for example, is quite teratogenic in mice, producing cleft palate, hydronephrosis, and a peculiar central nervous system lesion that results in the "waltzing syndrome" [20–22]. We believe it is inappropriate to induce rat liver hepatic microsomes with teratogenic chemicals that will thus be included in the embryo culture medium. Finally, microsomes contain more cytochrome P-450-based metabolizing ability per mg protein than do post-mitochondrial supernatant fractions and are preferable in an embryo culture system for that reason. For example, Fantel *et al* [17] report aminopyrine *N*-demethylase activities of 1.4 nmoles·(mg protein)$^{-1}$·min^{-1} in post-mitochondrial supernatant fractions, whereas we found enzyme activities of 7.3 nmoles·(mg protein)$^{-1}$·min^{-1} in microsomes.

Coupled microsomal-activating/embryo culture systems may be useful in assessing the role of maternal metabolism in embryo toxicity and teratology [16]. Considerable evidence suggests the importance of metabolism in reproductive toxicology and teratogenicity; however, only in systems such as this one can the importance of maternal versus embryonic metabolism be determined. For example, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is not only teratogenic [23, 24], but it also greatly alters female rat liver microsomal enzyme activities [25] and disturbs the sex hormone levels and reproductive ability of rhesus monkeys [26] at extraordinarily low doses.

The coupled microsomal-activating/embryo culture system has potential as a rapid, sensitive *in vitro* embryo toxicity and/or teratogenesis test. The sensitivity of this embryo culturing period, with metabolically active microsomes in the culture medium, may permit small amounts of drugs or chemicals to

be used, as well as smaller sample sizes, than *in vivo* toxicology or teratogenicity experiments. As little as 50 ng/ml of the alkylating agent 2,4,6-triethylenimino-1,3,5-triamine produces growth inhibition and characteristic morphological lesions in rat embryos [4]. In conclusion, the coupled microsomal-activating/embryo culture system will be useful both as a possible rapid *in vitro* embryo toxicity/teratogenicity test as well as an experimental system with which to study anomalous development (particularly that involving metabolism) *in vitro*.

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